

Markers of Inflammation in Bacterial Diarrhea among Travelers, with a Focus on Enteroaggregative *Escherichia coli* Pathogenicity

David E. Greenberg,¹ Zhi-Dong Jiang,² Robert Steffen,⁴
Mangala P. Verenker,⁵ and Herbert L. DuPont^{1,2,3}

¹Baylor College of Medicine, ²Center for Infectious Diseases, University of Texas–Houston, School of Public Health and Medical School, and ³St. Luke's Episcopal Hospital, Houston, Texas; ⁴University of Zurich Travel Clinic, Zurich, Switzerland; ⁵Goa Medical College, Bambolim, India

The intestinal inflammatory response of traveler's diarrhea acquired in Goa, India, and Guadalajara, Mexico, was studied. Fecal lactoferrin was found in stool samples in which enteroaggregative *Escherichia coli* (EAEC), enterotoxigenic *E. coli*, or *Salmonella* or *Shigella* species were isolated, with *Shigella*-positive cases showing the highest level. Samples from cases of *Shigella*-associated diarrhea had the highest concentrations of fecal cytokines. Travelers to India who had EAEC-associated diarrhea showed elevated levels of interleukin (IL)–8 (median, 341.15 pg/mL) and IL-1 β (median, 749.90 pg/mL). Although 15 travelers to Mexico who had EAEC-associated diarrhea had a median concentration of 0 pg/mL for both IL-8 and IL-1 β , 2 had high levels of IL-8 (1853 and 11,786 pg/mL), and 5 showed elevated levels of IL-1 β (1–1240 pg/mL). Samples from patients in India who had pathogen-negative diarrhea or from patients in Mexico who had asymptomatic EAEC infection were negative for cytokines. Bacterial pathogens causing traveler's diarrhea commonly produce intestinal inflammation, although a subset of patients with EAEC-associated diarrhea fail to develop an inflammatory response.

A group of organisms referred to collectively as “diarrheogenic *Escherichia coli*” is a major worldwide cause of illness in children and adults. In 1987, Nataro et al. [1] described a unique type of *E. coli* found among children with diarrhea in Santiago, Chile. These bacteria could be identified by their unique adherence to HEp-2 cells. The adherence pattern was described as aggregative. Although different patterns of attachment were identified, only the strains that had an aggregative adherence pattern were associated with diarrhea in these children. Enteroaggregative *E. coli* (EAEC) are now defined as *E. coli* that adhere to HEp-2 cells in an aggregative pattern and that do not secrete heat-stable or heat-labile enterotoxins [2]. Since first described, EAEC have become increasingly recognized as a leading cause of persistent childhood diarrhea in developing countries [1, 2]. EAEC have also been shown to cause diarrhea in travelers [3, 4], to cause foodborne outbreaks [5], and to be responsible for diarrhea in patients with AIDS [6] and in children attending day care centers [7].

The pathogenicity of EAEC is not completely understood. In one study of children, EAEC have been shown to produce fecal lactoferrin, interleukin (IL)–8, and IL-1 β , which are markers of

intestinal inflammation [8]. Whether EAEC regularly induce these or other cytokines has not been shown. In addition, it has not been shown whether these markers of inflammation become elevated in travelers who develop acute diarrhea and whether the cytokine inflammatory response relates to presence or absence of pathogen-specific diarrhea. Fecal lactoferrin, a marker of intestinal inflammation, has recently been shown by our group to be elevated in US adults with traveler's diarrhea acquired in Mexico and caused by EAEC [9]. Intestinal and systemic inflammation, including cytokine production, have been studied in diarrhea due to other bacteria, such as *Shigella* species [10, 11]. To see whether there were organism-specific patterns of inflammation with commonly encountered bacterial pathogens, we looked at intestinal markers of inflammation in adults with traveler's diarrhea in 2 widely separated regions of the world [12, 13].

Patients and Methods

Patient population. Stool samples were collected from adult European travelers with diarrhea that developed during visits to resort hotels in Goa, India, between 1996 and 1998 [12]. Stool samples were collected from US students who acquired diarrhea during short-term stays in Guadalajara, Mexico, during the summers of 1999 and 2000 [13]. Diarrhea was defined for both populations as the passage of ≥ 3 stools in 24 h, with at least 1 additional sign or symptom and a duration of ≤ 72 h. The signs or symptoms included abdominal pain or cramps, nausea, vomiting, fecal urgency, blood in stools, or increased intestinal gas. At both sites, patients provided a diarrheal stool sample for study. In Mexico, US students attending the same classes as case patients but who did not have enteric symptoms provided stool samples as control samples.

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Informed consent was obtained from all subjects, and institutional review board approval (University of Texas–Houston and University of Zurich) was obtained for the larger studies from which the samples used in this study were derived.

Reprints or correspondence: Dr. Herbert L. DuPont, St. Luke's Episcopal Hospital, 6720 Bertner Ave., MC 1-164, Houston, TX 77030 (hduPont@sluh.com).

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Stool analysis. Stool samples obtained from patients with diarrhea and from asymptomatic control subjects in Mexico were tested in our laboratories in Goa and Guadalajara for enteric pathogens, including enterotoxigenic *E. coli* (ETEC), EAEC, and *Shigella*, *Salmonella*, *Campylobacter*, *Aeromonas*, *Plesiomonas*, *Vibrio*, *Giardia*, *Entamoeba*, and *Cryptosporidium* species, by use of the same methods published elsewhere [14]. Three to 5 *E. coli* colonies from each subject were transported to Houston on peptone stabs for studies to determine whether they were ETEC or EAEC. Heat-stable and heat-labile enterotoxins of *E. coli* were detected with oligonucleotides labeled by T4 polynucleotide kinase and [32 P]-ATP [14]. EAEC were tested by the HEp-2 adherence assay [1, 3]. Stool samples were tested for lactoferrin by latex agglutination (Leuko-Test; Tech-lab). For cytokine and lactoferrin studies, stool samples were stored at -70°C until use. Aliquots of the original stool sample were diluted in PBS containing 2.5 $\mu\text{g/mL}$ leupeptin, 11 $\mu\text{g/mL}$ aprotinin, and 0.5 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (Sigma). After being thoroughly mixed and centrifuged (10 min at 10,000 g), the supernatants were tested, by ELISA, for the following cytokines: IL-8, IL-6, interferon (IFN)- γ , IL-1 β , IL-1 receptor antagonist (IL-1ra), and tumor necrosis factor (TNF)- α (Quantikine; R&D Systems). Stool lactoferrin and cytokine profiles were determined for patients positive for ETEC, EAEC, and *Salmonella* and *Shigella* species. Other enteric pathogens (e.g., miscellaneous bacteria and parasites) identified in small numbers were not included in the study. Only patients with single pathogens isolated from stool samples were included. Patients who had diarrhea but for whom no pathogen was identified (Goa) and asymptomatic persons for whom no pathogen was identified (Guadalajara) were included in the lactoferrin and cytokine studies as negative control subjects.

Statistical analysis. Fecal lactoferrin levels and cytokine concentrations were compared by the Kruskal-Wallis nonparametric group comparison. Data were analyzed with SAS, version 8 (SAS Institute).

Results

Fecal lactoferrin levels, graded as 0–4, were determined for the various study samples (figure 1). US subjects in Mexico who had asymptomatic EAEC infection, asymptomatic pathogen-negative travelers to Mexico, and travelers to India who had pathogen-negative diarrhea had median fecal lactoferrin levels of 0. The highest median level of lactoferrin, at 2, was found among travelers with *Shigella*-associated diarrhea acquired in India. Differences between groups were significant ($P = .0029$). Lactoferrin levels did not differ between patients with EAEC-associated diarrhea in India and those in Mexico ($P = .593$).

Fecal cytokine profiles were determined for patients with pathogen-specific diarrhea acquired in India (table 1). This included a study of cases of diarrhea due to EAEC (14 subjects), ETEC (44 subjects), and *Shigella* (11 subjects) and *Salmonella* (10 subjects) species. Cytokine results were compared with findings in diarrheal stool samples from patients for whom an enteric pathogen was not identified. Variations in the number of samples tested for each cytokine were due to availability of specimens.

The median concentration of fecal IL-6 for diarrhea due to EAEC, ETEC, and *Salmonella* species and for the pathogen-negative control samples was 0 pg/mL. Diarrhea due to *Shigella* species was associated with low levels of fecal IL-6 (9.64 pg/mL), which was significant when compared with the other groups studied ($P = .0001$). When compared in a pairwise fashion, IL-6 production in *Shigella*-associated diarrhea was not significantly different from that of the other diarrhea groups (for *Shigella* vs. ETEC, $P = .0825$; vs. EAEC, $P = .0558$; vs. *Salmonella*, $P = .1555$; and vs. no pathogen, $P = .1660$).

The median concentration of fecal TNF- α for diarrhea due to EAEC, ETEC, and *Salmonella* species and for the pathogen-negative control samples was 0 pg/mL. Diarrhea due to *Shigella* species was associated with a median TNF- α concentration of 2921.45 pg/mL, which was significant when compared with the other groups studied ($P = .0001$). When compared in a pairwise fashion, production of TNF- α in *Shigella*-associated diarrhea was statistically significantly different from that of the other diarrhea groups and the control group (for *Shigella* vs. ETEC, $P = .0001$; vs. EAEC, $P = .0009$; vs. *Salmonella*, $P = .0017$; and vs. no pathogen, $P = .0097$).

The median concentration of fecal IFN- γ for diarrhea due to ETEC and *Salmonella* species and for the control samples was 0 pg/mL. Production of fecal IFN- γ in patients with EAEC-associated diarrhea was 21.61 pg/mL. Diarrhea due to *Shigella* species was associated with a median IFN- γ concentration of 106.79 pg/mL. These amounts were not significantly different compared

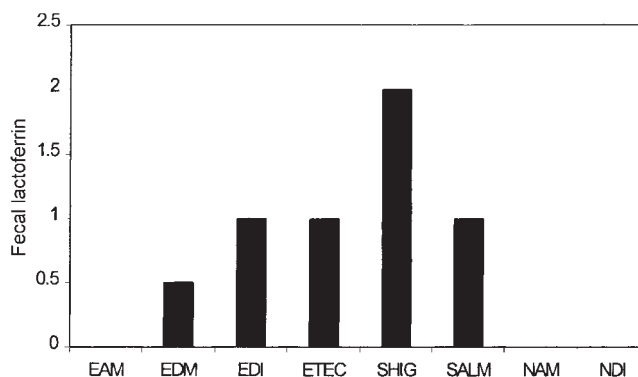


Figure 1. Fecal lactoferrin levels in adults with traveler's diarrhea, by pathogen and geographic location. Stool samples from control subjects and patients were tested for lactoferrin by latex agglutination. Groups tested included 10 travelers to Mexico who had asymptomatic enterotoxigenic *Escherichia coli* (EAEC) infection (EAM); 16 travelers to Mexico who had EAEC-associated diarrhea (EDM); 14 travelers to India who had EAEC-associated diarrhea (EDI); 44 travelers to India who had enterotoxigenic *E. coli*-associated diarrhea (ETEC); 11 travelers to India who had *Shigella*-associated diarrhea (SHIG); 10 travelers to India who had *Salmonella*-associated diarrhea (SALM); 13 asymptomatic travelers to Mexico for whom no pathogen was isolated (NAM); and 9 travelers to India who had pathogen-negative diarrhea (NDI). Agglutination was graded as follows: 0, no agglutination and therefore no lactoferrin present; 4, maximum agglutination. Bars represent median levels. Differences among groups were statistically significant ($P = .0029$, Kruskal-Wallis test).

Table 1. Fecal cytokines identified in samples from travelers with bacterial diarrhea acquired in Goa, India, 1996–1998.

Cytokine	EAEC		ETEC		<i>Shigella</i>		<i>Salmonella</i>		No pathogen identified (control samples)		P
	No. of samples tested	Median concentration, pg/mL	No. of samples tested	Median concentration, pg/mL	No. of samples tested	Median concentration, pg/mL	No. of samples tested	Median concentration, pg/mL	No. of samples tested	Median concentration, pg/mL	
IL-6	13	0	44	0	11	9.64	8	0	9	0	.0001
IL-8	14	341.15	43	315.16	11	3605.61	9	173.51	9	0	.0001
TNF- α	13	0	44	0	11	2921.45	10	0	9	0	.0001
IFN- γ	6	21.61	23	0	9	106.79	5	0	9	0	.0871
IL-1 β	14	749.90	43	97.91	11	792.96	9	35.39	9	0	.0304
IL-1ra	11	7161.05	41	1513.99	10	7645.48	6	291.38	9	218.68	.0001
IL-1 β :IL-1ra	11	0.099	41	0.10	10	0.10	6	0.01	9	0	.0001

NOTE. Variations in no. of samples tested were due to availability of specimens. EAEC, enteroaggregative *Escherichia coli*; ETEC, enterotoxigenic *E. coli*; IFN, interferon; IL, interleukin; IL-1ra, interleukin-1 receptor antagonist; TNF, tumor necrosis factor.

with the other groups studied ($P = .0871$). When compared in a pairwise fashion, concentrations of IFN- γ were not statistically significantly different in any of the diarrhea groups or in the control group (for EAEC vs. ETEC, $P = .4092$; *Shigella* vs. ETEC, $P = .1290$; *Salmonella* vs. ETEC, $P = .7350$; no pathogen vs. ETEC, $P = .3289$; EAEC vs. *Shigella*, $P = .3845$; EAEC vs. *Salmonella*, $P = .5258$; EAEC vs. no pathogen, $P = .1163$; *Shigella* vs. *Salmonella*, $P = .1607$; *Shigella* vs. no pathogen, $P = .0594$; and *Salmonella* vs. no pathogen, $P = .7080$).

The median concentration of fecal IL-8 for the control samples from the 9 subjects with pathogen-negative diarrhea was 0 pg/mL. IL-8 was found in samples from all pathogen-specific diarrhea groups, and the median concentrations (table 1) were significant when compared together as a group ($P = .0001$). When compared in a pairwise fashion, IL-8 production in *Shigella*-associated diarrhea was statistically significantly different from that of the other diarrhea groups and the control group. In addition, compared with that in control subjects with pathogen-negative diarrhea, IL-8 production in patients with diarrhea due to ETEC ($P = .0033$) or EAEC ($P = .0269$) was significant.

The median concentration of fecal IL-1 β for the control samples from the subjects with pathogen-negative diarrhea was 0 pg/mL. Samples from the subjects with pathogen-specific diarrhea showed elevated IL-1 β , compared with control samples (table 1). When compared in a pairwise fashion, only IL-1 β production in patients with *Shigella*-associated diarrhea was statistically significantly different from that of the other diarrhea groups and the control group (for *Shigella* vs. ETEC, $P = .0032$; vs. EAEC, $P = .0486$; vs. *Salmonella*, $P = .0034$; and vs. no pathogen, $P = .0018$); group comparison showed statistical differences as well ($P = .0304$).

IL-1ra was found in samples from all groups studied, including the pathogen-negative control subjects with diarrhea (table 1). When compared in a pairwise fashion, IL-1ra production in *Shigella*-associated diarrhea was statistically significantly different from that of the other diarrhea groups and the control group (for *Shigella* vs. ETEC, $P = .0001$; vs. EAEC, $P = .0267$; vs. *Salmonella*, $P = .0001$; and vs. no pathogen, $P = .0001$). In

addition, IL-1ra production in *Salmonella*-associated diarrhea was significantly different from that in the pathogen-negative control group ($P = .0015$). IL-1ra production in EAEC-associated diarrhea was significantly different from that in *Salmonella*-associated diarrhea as well ($P = .0343$). Group comparison showed statistical significance ($P = .0001$). The ratio of IL-1 β to IL-1ra also showed statistical significance ($P = .0001$). The IL-1 β :IL-1ra ratios are given in table 1.

Fecal cytokine levels were tested for subjects traveling to Mexico. The median concentrations of fecal IL-6, TNF- α , and IFN- γ were 0 pg/mL for all groups studied. Samples from travelers to Mexico who had EAEC infection had median concentrations of 0 pg/mL for fecal IL-6, IL-8, TNF- α , IFN- γ , and IL-1 β regardless of whether the subjects had diarrhea. Samples from the asymptomatic pathogen-negative control subjects had median concentrations of 0 pg/mL for the same fecal cytokines. The median IL-1ra concentration was 99.49 pg/mL in stool samples from travelers to Mexico with asymptomatic EAEC infection, 156.34 pg/mL in stool samples from travelers to Mexico who had EAEC-associated diarrhea, and 53.65 pg/mL in samples from asymptomatic pathogen-negative travelers to Mexico.

Fecal IL-8 concentrations among the various groups (India or Mexico) with EAEC infection are shown in figure 2. Samples from travelers to Mexico who had asymptomatic EAEC infection, asymptomatic pathogen-negative subjects in Mexico, and pathogen-negative patients with diarrhea in India had median IL-8 concentrations of 0 pg/mL. IL-8 was not detected in individual samples from the latter 2 groups; however, among travelers to Mexico who had EAEC-associated diarrhea, 2 patients were positive for IL-8, with concentrations of ~1853 pg/mL and 11,786 pg/mL, which represented the highest IL-8 concentration in the study. Samples from travelers to India who had EAEC-associated diarrhea had a median concentration of 341.15 pg/mL. Concentrations of IL-8 ranged from 0 to >4000 pg/mL, with the highest levels found in patients with EAEC-associated diarrhea.

Fecal IL-1 β concentrations in the various subject groups are shown in figure 3. Samples from asymptomatic travelers to

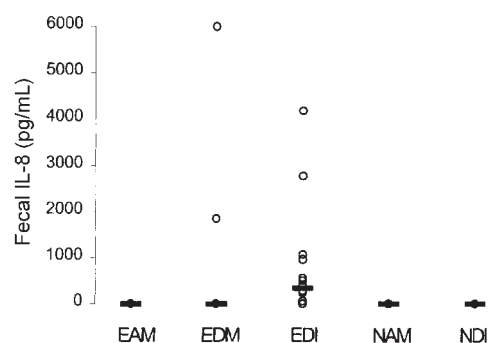


Figure 2. Fecal interleukin (IL)–8 levels in adult travelers to Goa, India, and Guadalajara, Mexico, who had enteroaggregative *Escherichia coli* (EAEC) infection. Groups tested included 9 travelers to Mexico who had asymptomatic EAEC infection (EAM); 15 travelers to Mexico who had EAEC-associated diarrhea (EDM); 14 travelers to India who had EAEC-associated diarrhea (EDI); 11 asymptomatic travelers to Mexico for whom no pathogen was isolated (NAM); and 9 travelers to India who had pathogen-negative diarrhea (NDI). Symbols represent individual samples; horizontal lines represent median concentrations. Differences among groups were statistically significant ($P = .003$, Kruskal-Wallis test).

Mexico who had EAEC infection had a median fecal IL-1 β concentration of 0 pg/mL, with 1 sample producing ~1180 pg/mL. Samples from US travelers to Mexico who did not have diarrhea or enteric pathogens had a median concentration of 0 pg/mL, with 3 stool samples producing 2–90 pg/mL. Samples from travelers to India who had pathogen-negative diarrhea had a median concentration of 0 pg/mL, with 5 samples producing 60–1270 pg/mL. Samples from travelers to Mexico who had EAEC-associated diarrhea had a median concentration of 0 pg/mL, with 5 samples producing 1–1240 pg/mL. Samples from travelers to India who had EAEC-associated diarrhea had a median concentration of 749.90 pg/mL (range, 0 to >800 pg/mL). The 2 patients in Mexico who had EAEC-associated diarrhea and the highest levels of IL-8 (1853 and 11,786 pg/mL) also showed high levels of fecal IL-1 β (1183 and 1110 pg/mL, respectively) and maximum (3) levels of lactoferrin.

Discussion

In a published study of patients with shigellosis, immunohistochemical staining of rectal biopsy samples was done to look for elevated concentrations of multiple cytokines. Increases in levels of IL-1, IL-6, IFN- γ , and TNF- α were seen in samples that had severe inflammation histologically [10]. Another study showed that infection with *Shigella* was associated with the release of a number of cytokines in the plasma and stool, including TNF- α , IL-1 β , IL-1 α , IL-6, IL-8, and IFN- γ [11]. In the present study, we examined these cytokines in patients with traveler's diarrhea. Previous studies have shown that fecal lactoferrin is a sensitive marker for intestinal inflammation [15]. We have shown a correlation between EAEC infection and elevated levels of fecal lactoferrin but not elevated levels of fecal leukocytes

or frequency of stool samples testing positive by Hemocult [9]. In this study, we looked at fecal lactoferrin levels in the various study groups to determine the relationship between this marker of intestinal inflammation and fecal levels of cytokines. We were particularly interested in patients with EAEC infection and diarrhea, to further our understanding of the pathogenicity of this pathogen.

In the present study, symptomatic *Shigella* infection was associated with the most intense response in bacterial diarrhea, when we looked at the associated fecal lactoferrin and cytokine levels. IL-6, TNF- α , and IFN- γ levels were not found to be elevated in samples from non-*Shigella*-associated diarrhea caused by ETEC, *Salmonella*, or EAEC. Elevated levels of fecal IL-8 and IL-1 β were found in samples from European travelers to Goa, India, with symptomatic bacterial infection. EAEC-associated diarrhea occurring in India was associated with increased fecal levels of IL-8, IL-1 β , and IL-1 α , as was seen for travelers to India who had ETEC diarrhea, shigellosis, or salmonellosis. Samples from subjects with pathogen-negative diarrhea in India were negative for these inflammatory products, showing an important relationship between intestinal inflammation and diarrhea due to defined bacterial pathogens in this group of travelers. A specific pattern of cytokine activation may distinguish specific enteric infection, producing a pathogen signature. Future study of the cytokine response of organism-specific infection is needed. Of interest, a majority of the US students with diarrhea acquired in Mexico were negative for inflammatory markers, even when infected with EAEC strains.

This study demonstrated that diarrhea in international travelers with EAEC infection may or may not be associated with

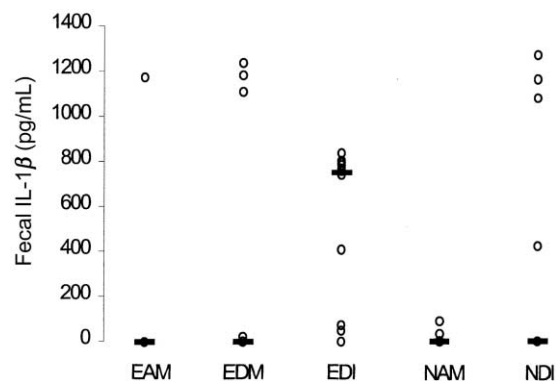


Figure 3. Fecal interleukin (IL)–1 β levels in adult travelers to Goa, India, and Guadalajara, Mexico, who had enteroaggregative *Escherichia coli* (EAEC) infection. Groups tested included 10 travelers to Mexico who had asymptomatic EAEC infection (EAM); 16 travelers to Mexico who had EAEC-associated diarrhea (EDM); 14 travelers to India who had EAEC-associated diarrhea (EDI); 13 asymptomatic travelers to Mexico for whom no pathogen was isolated (NAM); and 9 travelers to India who had pathogen-negative diarrhea (NDI). Symbols represent individual samples; horizontal lines represent median concentrations. Differences among groups were statistically significant ($P = .0098$, Kruskal-Wallis test).

intestinal release of markers of inflammation. Steiner et al. [8] previously showed that fecal IL-8, IL-1 β , and lactoferrin could be identified in samples from Brazilian children infected with EAEC. Our data from a study of travelers to India who had EAEC-associated diarrhea resembles findings for those Brazilian children. In the study by Nataro et al. [1], patients with EAEC infection had higher ratios of IL-1 β to IL-1ra than did uninfected control subjects. This presumably represents more IL-1 receptor activation in these patients.

One possible explanation for variation in intestinal markers of inflammation with EAEC-associated diarrhea is that not all strains are pathogenic. Variation in virulence of EAEC has been demonstrated in healthy volunteers given different strains [16]. Varying levels of virulence have also been shown in Nigerian children with EAEC infection [17]. In the present study, there were apparent differences in fecal lactoferrin concentrations and cytokine production between most of the travelers to India who had EAEC-associated diarrhea and a majority of the travelers to Mexico who had EAEC-associated diarrhea. A number of plasmid-encoded virulence factors have been recently identified that appear to at least partially explain the pathogenic potential of EAEC strains. These include the fimbriae AAF/I and AAF/II, which are thought to play a role in mucosal adherence [18]; Pet (plasmid-encoded toxin) [19, 20]; EAST-1 (heat-stable enterotoxin) [21]; Shet1 and Shet2 (*Shigella* enterotoxins) [22]; AspU, which is a cryptic secreted protein [23]; and fliC, which is thought to trigger IL-8 release [24]. We currently are examining the EAEC strains isolated from travelers to India and Mexico, to determine whether the presence or absence of defined virulence properties correlates with different levels of inflammation, as tested by fecal cytokine analysis. Host factors may be important in the pathogenesis of EAEC-associated diarrhea. Children from developing countries, where malnutrition is common, show a more intense cytokine response and develop growth and developmental abnormalities associated with EAEC infection [8]. The major difference between our 2 study populations was country of origin (i.e., Europe vs. the United States). The illness severity in the 2 populations was comparable.

Differences in markers of inflammation between patients studied in India and those studied in Mexico could be explained by geographic variations in organism virulence. Despite the differences in median concentrations of inflammatory markers in the 2 settings, individual patients in Mexico who had EAEC infection had inflammatory diarrhea: 2 of 15 patients with EAEC-associated diarrhea from Mexico produced IL-8 (including the subject with the highest concentration in either setting), and 5 of 16 patients produced detectable levels of IL-1 β . Of note, the 2 patients who produced IL-8 also produced large amounts of IL-1 β . The production of IL-8 also seemed to be related to the production of lactoferrin. The 2 patients from Mexico who had EAEC-associated diarrhea with increased fecal IL-8 produced 3 levels of lactoferrin, which were the highest recorded.

Asymptomatic EAEC infection is extremely common in US travelers to Mexico [13], and EAEC strains commonly can be found in foods served at public restaurants in this setting [25]. The common occurrence of asymptomatic EAEC infection in travelers to Mexico and the lack of intestinal inflammatory markers raise questions about pathogenicity in these cases. Another way to approach determination of the pathogenic potential of a bacterial isolate in enteric infection is through documentation of the intestinal secretory IgA (sIgA) response to the organism. We have previously shown that virulent organisms induce a specific sIgA response during infection, whereas less pathogenic strains do not [26]. We plan to study the sIgA response to homologous EAEC infecting the patients with cytokine-positive and cytokine-negative diarrhea, to indirectly evaluate virulence of the organism. We have previously found in a small study that approximately one-half of patients with EAEC-associated diarrhea developed an sIgA response to their infecting organism [27]. We believe that, through characterization of fecal cytokines, EAEC virulence properties, and sIgA responses to infecting strains, we will further our understanding of the pathogenesis of EAEC-associated diarrhea.

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